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Muscarinic Agonists as Insecticides and Acaricides

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Abstract: A series of known agonists of the mammalian muscarinic receptor were prepared and evaluated for their insecticidal potential. It was discovered that pests such as *Nilaparvata lugens* (brown planthopper), *Nephotettix cincticeps* (green leafhopper), *Tetranychus urticae* (two-spotted spider mite) and *Aphis gossypii* (cotton aphid) were particularly sensitive to most of these compounds. Several analogs proved to be extremely active, surpassing commercial standards in some of the laboratory bioassays. These compounds exhibited a range of potencies for the insect (*Musca*) muscarinic receptor. Addition of GTP significantly reduced the affinity of the most potent analog for the *Musca* mAChR, indicating the compound functions as an agonist in insect tissue. Regression analysis indicated that significant relationships exist between displacement of [^{3}H]QNB at the *Musca* muscarinic receptor and whole organism toxicity to three insect and one mite species. The results suggested that the insect muscarinic receptor represents a viable target site for insecticidal action.

Key words: muscarinic, agonist, insecticide, acaricide

1 INTRODUCTION

One of the major issues facing insect management today is resistance to current insecticides.[1] Such loss of sensitivity can be the result of a number of mechanisms, including the continued selection of pests possessing a metabolic pathway favorable for the degradation of the toxic agent. However, it can also arise from the selection of pests bearing a mutation at the target site which, at least in part, is responsible for toxicity.[2] Consequently, the development of new insecticides becomes a complicated task, since new agents using previously exploited biochemical targets rapidly lose efficacy in the field as a result of cross-resistance. Therefore, the discovery of a compound or a class of compounds which operates *via* a novel mode of action would prove extremely valuable and is the primary goal of our current research.

Upon review of the literature, we felt that an opportunity for a unique mode of action might exist at the acetylcholine regulated muscarinic receptor complex (mAChR). Cholinergic receptors fall into two main classes, muscarinic and nicotinic (nAChR) receptors. Nicotinic receptors, which belong to a family of receptors known as ligand-gated ion channels, have already proven to be a viable target for insect control. This is the major site of action for the commercial dithiazolines (cartap)[3] and the nitromethylene heterocycles (imidacloprid).[4] On the other hand, there is minimal information on the potential of the mAChR as a viable target site for insect control.[5,6] These receptors belong to a different superfamily of proteins, generally referred to as G-coupled proteins. Unlike the nAChR, muscarinic receptors do not contain integral ion channels but rather transduce their signals intracellularly *via* second messengers (such as cAMP) through interactions with G-coupled proteins. The overall effect is that ion channels in the membrane selective for K^+, Ca^+ and Cl^- are indirectly opened or closed by a series of events which occur when acetylcholine or other agonists bind to the mAChR.[7]

The existence of muscarinic receptors in various insect species has been known for some time.[8–19] However, detailed pharmacology in these and other insects remained relatively unexplored. This might be due to the fact that in the insect CNS, nicotinic receptors predominate over muscarinic ones.[3] Recently, several attempts have been made to better understand the pharmacology of muscarinic receptors in insects.[13–19] One important finding was that a cloned

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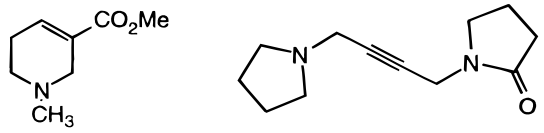


1 2

Fig. 1. Compounds discussed. **1** = Arecoline; **2** = Oxotremorine.

and sequenced *Drosophila* mAChR exhibited remarkable homology with the corresponding mammalian (rat cortex) mAChR in the transmembrane portion of the protein.[17,18]

Early studies in our laboratories revealed that known muscarinic agonists such as arecoline (Fig. 1; **1**) and oxotremorine (**2**), induced rapid convulsions when injected into third-instar *Heliothis virescens* F. larvae (C. P. Chang, unpublished). However, these compounds were lethal only at high doses and lacked contact activity. This observation provided the first indication that the muscarinic site might be a viable target site for insect control and this led to our interest in muscarinic compounds which exhibited selectivity for the insect mAChR.

We were intrigued by more recent reports that have appeared in the medicinal literature of highly potent agonists for the mammalian mAChR.[20,21] The most active of these were analogs derived from arecoline, in which the ester moiety is replaced by a more stable bioisostere, typically a heterocycle. In addition to being more hydrolytically stable than the ester moiety, the heterocycle apparently affords increased affinity at the receptor.[21] Modification of the tetrahydropyridine also had profound effects. This portion of the molecule most significantly affects the relative conformation of the pharmacophores as well as the pKa (an important parameter of transport).[22] In the report below, we describe the insecticidal activity for some of these agonists.

2 EXPERIMENTAL

2.1 Synthesis

2.1.1 Oxadiazoles

Oxadiazoles **3**–**7**,[23] **8**,[24] **9**–**11**[25] and **12**[26] were prepared according to published procedures. In general, they were prepared either by reaction of acetamide oxime or *N*-hydroxyguanidine in refluxing ethanol with the appropriate amino ester. Compound **13** was derived from 1-azabicyclo[3.2.1]octan-3-one[27] by carbon homologation[28] and subsequent treatment with acetamide oxime.

2.1.1.1 exo-6-[5-(3-Methyl-1,2,4-oxadiazol)-yl]-1-azabicyclo[3.2.1]octane (***13***). To 1-trimethylsilyl-1,3-dithiane (2·13 g, 11·0 mmol) in anhydrous tetrahydrofuran (THF; 20 ml) under nitrogen at −40°C was added *n*-butyl lithium in hexane (2·5 M; 4·4 ml; 11·0 mmol) and the resulting solution was allowed to stir at this temperature for 2 h. To this was then added a solution of 1-azabicyclo[3.2.1]octan-3-one[14] (1·10 g, 9·0 mmol) in THF (20 ml) at a rate such that the internal temperature of the reaction did not exceed −30°C. After addition was complete, the reaction mixture was allowed to warm to room temperature and stir overnight. The mixture was quenched with water and extracted into dichloromethane. The organics were combined, dried over sodium sulfate, filtered and evaporated. The crude dithiane (2·37 g) was dissolved in methanol and hydrogen chloride was passed into the solution until saturated. The resulting mixture was heated at 55°C for 24 h. The mixture was cooled to room temperature and concentrated under vacuum. The residue was partitioned between 1 M sodium carbonate and dichloromethane and, following separation, the aqueous layer was extracted with two additional portions of dichloromethane. The organics were combined, dried over sodium sulfate, filtered and evaporated under reduced pressure. Distillation (65°C at 0·3 mm Hg) provided 3-carbomethoxy-1-azabicyclo[3.2.1]octane as a single diastereomer (750 mg, 50%): [^{1}H]NMR deuterochloroform, 400 MHz) δ 3·59 (s, 3H), 2·99 (dd, 1H), 2·80–2·90 (m, 3H), 2·76 (ddd, 1H), 2·70 (dddd, 1H), 2·52 (ddd, 1H), 2·23–2·27 (m, 1H) and 1·59–1·79 (m, 4H). [^{13}C]NMR (deuterochlorofom, 400 MHz) δ 174·9, 60·0, 56·9, 51·7, 51·5, 36·5, 33·6, 33·2 and 30·2. *m/e* 169 (M^+). The ester (1·07 g, 6·3 mmol) was then added to a suspension containing powdered 3 Å molecular sieves (6 g) and acetamide oxime (2·8 g, 38·0 mmol) in a solution of sodium ethoxide (0·76 M, 50 ml). The resulting mixture was refluxed under a nitrogen atmosphere for 2 h. The reaction mixture was then cooled to room temperature and the solvent removed under reduced pressure. The residue was taken up into dichloromethane filtered through a pad of celite, and concentrated under reduced pressure. The residue was purified by flash chromatography [alumina; dichloromethane + methanol (99 + 1 by volume)] followed by kugelrohr distillation (80–85°C at 0·02 mm Hg) to provide 0·65 g of the title compound as a clear oil. [^{1}H]NMR (deuterochloroform, 400 MHz) δ 3·25 (dddd, 1H), 3·04 (dd, 1H), 2·84–2·93 (m, 3H), 2·78 (ddd, 1H), 2·51 (bd, 1H), 2·20–2·30 (m, 1H), 2·23 (s, 3H), 1·80–1·90 (m, 2H), 1·70–1·80 (m, 1H) and 1·60–1·70 (m, 1H). [^{13}C]NMR (deuterochlorform) δ 180·6, 166·8, 59·8, 57·8, 51·5, 34·8, 33·0, 30·0, 29·7 and 11·3. Anal. Calc'd for $C_9H_{14}N_4O$ (194·2) C, 55·65; H, 7·27; N, 28·84. Found: C, 55·04; H, 7·55; N, 28·82.

2.1.2 Tetrazoles/triazoles

Triazole **14** and tetrazoles **15**–**17** were prepared according to published procedures.[25] Tetrazole **18** was afforded on reaction of *endo*-3-methanesulfonyloxy-1-azabicyclo[2.2.1]-heptane[25] with 5-(methylthio)tetrazole.

2.1.2.1 (±)*exo-3-[5-(Methylthio)tetrazol-2-yl]-1-azabicyclo[2.2.1]heptane* (***18***). To a suspension of sodium hydroxide (0·95 g of a 60% oil dispersion, 23·8 mmol) in *N*,*N*-dimethylformamide (DMF, 20 ml) was added 5-(methylthio)tetrazole (2·75 g, 23·7 mmol). After hydrogen evolution had ceased (30 min), a solution of *endo*-3-methanesulfonyloxy-1-azabicyclo[2.2.1]-heptane[25] (1·0 g, 5·2 mmol) was added and the resulting mixture was refluxed for 2 h. The mixture was cooled and the solvent evaporated under reduced pressure. The residue was partitioned between 1 M sodium carbonate and dichloromethane and, following separation, the aqueous layer was extracted with two additional portions of dichloromethane. The organics were combined, dried over sodium sulfate, filtered and evaporated under reduced pressure. Flash chromatography [alumina; hexane + ethyl acetate (1 + 1 by volume)] provided the title compound as a yellow oil in 31% yield. [^{1}H]NMR (deuterochloroform) δ 4·54 (ddd, 1H), 3·28 (ddd, 1H), 3·10 (ddd, 1H), 3·02 (bd, 1H). 2·99 (bd, 1H), 2·85 (m, 1H), 2·61 (s, 3H), 2·47 (m, 1H), 2·42 (bd, 1H), 1·72–1·77 (m, 1H) and 1·26–1·30 (m, 1H). [^{13}C]NMR (deuterochloroform) δ 164·7, 66·3, 61·8, 58·3, 53·6, 43·6, 27·9 and 14·4. *m/e* 180 (M^+).

2.2 Biology

Laboratory bioassays were run against *Nilaparvata lugens* (Staol) (brown planthopper), *Nephotettix cincticeps* (Uhl.) (green leafhopper), *Tetranychus urticae* Koch (two-spotted spider mite) and *Aphis gossypii* (Glov.) (cotton aphid).

2.2.1 Rice foliar contact assay—Nilaparvata lugens (*brown planthopper*) *and* Nephotettix cincticeps (*green leafhopper*)

Compounds were dissolved in reagent-grade acetone and diluted further in water to yield the desired concentrations. The acetone concentration in the aqueous solvent solution was 125 ml litre^{-1}. Rice seedlings were prepared by washing soil from the roots. Circles of metal screen were prepared and a slit was cut from the outer edge to the center. Four rice seedlings were slipped through the slit in the screen and then placed in glass cups filled with water. Glass cylinders which fitted on the glass cups were placed over the metal screen to secure it and the cylinder and cup were taped together. One-half (0·5) ml of the aqueous solvent solutions was sprayed into each cylinder using a DeVilbiss-type nozzle. Generally, four cylinders were treated at each concentration. Three hours after spraying, five third-instar green leafhopper or brown planthopper nymphs were aspirated into each cylinder. The cylinders were capped with screened lids, placed in racks, and held in a controlled environment chamber at 28°C, 75% RH and a 14 : 10 h light : dark photoperiod. Mortality was assessed at 48 h after treatment. Percentage mortality was corrected for check mortality using Abbott's formula[29] and LC_{50} values were determined using probit analysis.[30]

2.2.2 Squash cotyledon contact assay—Aphis gossypii (*cotton aphid*)

Yellow crookneck squash seedlings in the expanded cotyledon stage were trimmed to one cotyledon and infested with cotton aphid nymphs and adults 16 to 24 h before treatment. Compounds were dissolved in HPLC-grade acetone and diluted in aqueous surfactant (0·5 g litre^{-1} 'Tween'-20) to yield the desired series of finished concentrations. The concentration of solvent in the aqueous solvent/surfactant solution was 12·5 ml litre^{-1} or less. The infested cotyledons were dipped in the aqueous solvent/surfactant solution. Generally, three seedlings were treated at each of five concentrations. The treated plants were held in a controlled environment chamber at 27°C, ambient RH and a 16 : 8 h light : dark photoperiod. Mortality was assessed at 72 h after treatment by making a visual estimate of the reduction in aphid numbers relative to control plants treated with a solvent blank. An eight-point scale was used: 0, 50, 70, 80, 90, 95, 99 and 100% control. LC_{50} values were determined using the trimmed Spearman–Karber procedure.[31]

2.2.3 Squash cotyledon contact assay—Tetranychus urticae (*two-spotted spider mite*)

Fully expanded squash cotyledons were infested with a mixed population of two-spotted spider mites by applying infested leaf sections bearing 10–20 adults to the upper leaf surface. The donor leaf was removed after 24 h. Compounds were dissolved in a solution of 88·75% acetone, 8·0% m-Pyrol, 2·0% Exxon 200 and 1·12% 'Tween' 20. This solution was diluted with aqueous solvent-surfactant solution to yield the desired series of concentrations (a four-fold dilution series was generally used). The infested cotyledons were sprayed to run-off by applying 0·5 ml of aqueous solution to each leaf surface with a syringe tipped with a TN-2 nozzle. Four plants were treated at each concentration and eight plants were treated with an aqueous solvent/surfactant blank as controls. The treated plants were held in a controlled environment chamber at 26°C, *c*.75% RH and 12 : 12 h light : dark photoperiod. Live adult female mites were counted 72 h after application and percentage reduction relative to the aqueous solvent/surfactant-treated check was calculated. LC_{50} values were determined using the trimmed Spearman–Karber procedure.[31]

2.3 Biochemistry

2.3.1 [^{3}*H*]*QNB binding assay*

Heads from adult houseflies, *Musca domestica* L., were separated by freezing intact flies, followed by differential sieving. Washed membranes were prepared from these

frozen heads and protein aliquots were stored at −80°C for no more than two months. Binding activity was unchanged during this storage period. A binding buffer containing 50 mM Tris.HCl, 120 mM sodium chloride and 2 mg ml^{-1} bovine serum albumen (BSA) at pH 7·4 was used for all dilutions including protein, competing compounds and radioligand.[14] All stock solutions of compounds were made in dimethyl sulfoxide (DMSO) and diluted with binding buffer to a final concentration of DMSO of no greater than 10 ml $litre^{-1}$. All experiments were conducted in triplicate. As determined by protein linearity experiments, 300 μg of *Musca* neural protein was used per sample. [^{3}H]QNB (quinuclidinyl benzilate) was purchased from NEN DuPont (Wilmington, DE) with specific activity of 43 Ci $mmol^{-1}$. Based on the K_d obtained from the Scatchard analysis, all experiments were conducted in the presence of 2 nM [^{3}H]QNB. Non-specific binding was defined in the presence of 10 μM atropine. Samples were incubated at 25°C in a shaking water-bath and the reaction was terminated after 2 h by filtration in a Brandel 96-cell harvester. Whatman GF/C filter mats presoaked with the binding buffer containing 10 mg ml^{-1} BSA were used for the filtration. The filters were rapidly washed with cold binding buffer and counted in a Beckman LS 6000IC liquid scintillation counter, using Ultima Gold scintillation cocktail (Packard). Under these conditions, specific binding comprised 85–90% of total binding. The K_d and B_{max} were determined to be 0·51 ± (0·25) nM and 59·6 ± (13·57) fmol mg^{-1}, respectively.

Cmpd	R
3	Me
4	Et
5	NH_2
6	Cl
7	Br

Fig. 2. Compounds referred to in text.

3 RESULTS AND DISCUSSION

3.1 1,2,4-Oxadiazoles

3.1.1 3′-Substituent effects

An initial set of quinuclidine-based agonists of the mammalian mAChR, employing a 1,2,4-oxadiazole as the ester bioisostere, was chosen from the literature to test the viability of insect control *via* interaction at the insect mAChR (Fig. 2). Biological results were promising (Table 1). Good broad-spectrum control was afforded by methyl and amino oxadiazoles **3** and **5**. Better cotton aphid activity was observed for halogen-substituted analogs **6** and **7**, but it was offset by a loss in activity against the other test species. Of interest was the loss of activity afforded on exchange of an ethyl for a methyl substituent at the 3′-position. This is not only consistent with the corresponding binding affinity for the insect (*Musca*) mAChR, it coincides with conclusions drawn in the literature for the mammalian (rat cortex) mAChR.[21] Data from those studies indicated that binding-site affinity was maximized by small, electron-donating substituents at the 3′-position of the oxadiazole. Since the optimal substituents from those studies were identified as methyl and amino, we shifted our focus toward maximizing insecticidal activity *via* the amine-containing ring.

3.1.2 Amine modifications

Using 3-methyl-1,2,4-oxadiazole as the model bioisostere, we investigated several different amine replacements for the quinuclidine (Fig. 3). This part of the molecule serves as a 'scaffold' which defines a particular relative orientation of the amine (protonated at physio-

TABLE 1
Insecticidal Activity and In-vitro Receptor Binding Activity of 1-Azabicyclo[2.2.2]octanes (Quinuclidines)

		LC_{50} (*mg litre*$^{-1}$)				K_i (μM)[b]
Cmpd	*R*	*BPH*[a]	*GLH*	*TSSM*	*CA*	Musca
3	Me	4·6	29	3·1	11	0·62
4	Et	119	220	14	34	1·42
5	NH_2	11	73	7·3	5·0	0·89
6	Cl	21	133	19	9·6	0·25
7	Br	—[c]	—[c]	105	3·2	0·61
Ethofenprox		0·13	0·06	—	—	—
Chlorpyrifos		—	—	—	2·2	—
Propargite		—	—	19	—	—

[a] BPH: brown planthopper, GLH: green leafhopper, TSSM: two-spotted spider mite, CA: cotton aphid.
[b] QNB Binding; data are the mean of triplicates with SEM < 15%.
[c] Not tested.

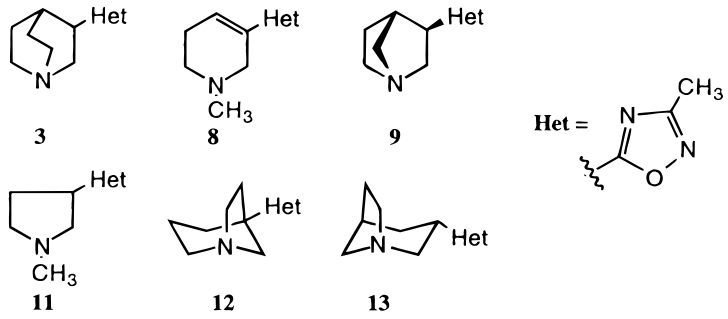


Fig. 3. Compounds referred to in text.

logical pH) with respect to the oxadiazole. The insecticidal data of several 3-methyl-1,2,4-oxadiazole amines are summarized in Table 2. Most obvious is the outstanding activity afforded by the 1-azabicyclo[2.2.1] heptane system (compound **9**). This was not a surprise since azanorbornane (**9**; Het = H) exhibited outstanding affinity for the insect receptor and was among the most potent and efficacious agonists reported in the mammalian literature.[21] Apparently, the 1-azanorbornyl framework allows for the optimal geometry of the pharmacophores.[22] It has been proposed that the active conformation for the mammalian site occurs at a C2–C3–C5′–O1′ dihedral angle of 274° (Fig. 4).[32] Unlike the other amine systems, this conformation in the azanorbornyl system is less than 1 kcal mol^{-1} above the global minimum and thus is readily accessible.[33] Also, since there is nearly free rotation about the C3–C5′ bond, the azanorbornyl moiety possesses the flexibility necessary to undergo a conformational change. This supposedly occurs as the insecticide molecule passes from a low-affinity, functionally inactive binding site of the receptor to a distinct, high-affinity functionally active binding site in the same receptor complex.[21]

3.2 Tetrazoles

Having established the outstanding insecticidal potential of putative agonists acting at the insect muscarinic site, we began to investigate heterocyclic alternatives to the 1,2,4-oxadiazole. Among several that were investigated, we found two heterocycles, a 1,2,4-triazole and a tetrazole, which exhibited particularly interesting insect activity (Fig. 5). The insect data for the respective

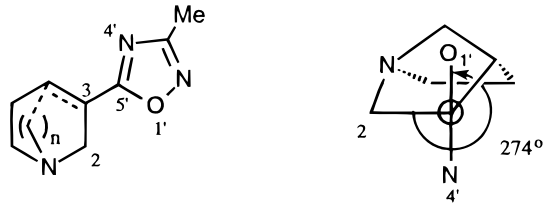
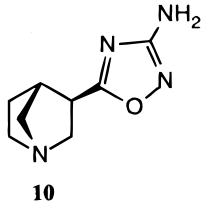


Fig. 4. Proposed active conformation of muscarinic agonists.

TABLE 2
Insecticidal Activity and In-vitro Receptor Binding Activity of 3-Methyl-1,2,4-Oxadiazolyl-Amines

		LC_{50} (*mg litre*$^{-1}$)				K_i (μM)[b]
Cmpd	*Amine*	*BPH*[a]	*GLH*	*TSSM*	*CA*	musca
3	Quin[c]	4·6	29	3·1	11	0·62
8	THP[d]	4·1	14	185	50	7·40
9	[2.2.1][e]	0·03	1·6	0·2	0·4	0·086
11	Pyrrolidine	>400[h]	>400[h]	50	>400[h]	~100[i]
12	6-[3.2.1][f]	4·9	45	10	6·4	1·16
13	4-[3.2.1][g]	19	140	20	200	7·00

[a] As Table 1.
[b] QNB Binding; data are the mean of triplicates with SEM < 15%.
[c] 1-azabicyclo[2.2.2]octane.
[d] tetrahydropyridine.
[e] *exo*-1-azabicyclo[2.2.1]heptane.
[f] 6-(subst)-1-azabicyclo-[3.2.1]octane.
[g] 4-(subst)-1-azabicyclo[3.2.1]octane.
[h] < 10% control at top rate.
[i] 45% inhibition at 100 μM.



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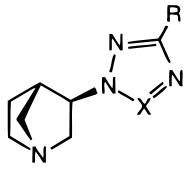

Cmpd	X	R
14	CH	NH_2
15	N	NH_2
16	N	H
17	N	Br
18	N	SMe

Fig. 5. Compounds discussed in text.

amino-substituted analogs **14** and **15** are compared against those of the corresponding 3-amino-1,2,4-oxadiazole **10** in Table 3. In each case the *exo* diastereomer is represented. As can be seen from the data, the tetrazole appeared to be superior to the triazole and nearly equivalent to the oxadiazole as an efficacious ester bioisostere. The in-vivo results are consistent with the relative affinities for the insect (*Musca*) mAChR. Several other tetrazoles also proved to exhibit noteworthy insecticidal activity (Table 3). In particular, hydrido and bromo analogs **16** and **17** gave analogous results to **15** against brown planthopper, green leafhopper and two-spotted spider mite with somewhat reduced (especially in the case of **16**) activity against cotton aphid.

3.2 Mode of action considerations

Establishing the mode of action for a particular biological response generally requires three key elements: (1) demonstrating an in-vitro interaction with a particular receptor site or enzyme (including the lack of affinity for other, related sites) with a range of potencies for a series of compounds, (2) establishing the nature of that interaction (e.g. agonist/antagonist) and (3) demonstrating a relationship between in-vitro potency for the receptor and in-vivo efficacy (e.g. mortality). As seen in Tables 1–3, the compounds in this study showed a range of potencies at the *Musca* muscarinic binding site. K_i values ranged from 0·028 to >100 μM in the three series tested. Coupled with this, none of the compounds in the study significantly displaced the nicotinic antagonist, [^{3}H]α-bungarotoxin in the same tissue preparation (unpublished). Overall, the most active analog, both *in vitro* and *in vivo*, was compound **9**. Although **9** was reported to be an agonist in mammalian tissue[20,21] and exhibited a high potency (K_i = 86 nM) for the insect (*Musca*) [^{3}H]QNB binding site, it was not clear whether this compound was acting as an agonist or an antagonist in the insect tissue. Consequently, competition curves were generated for carbachol and **9** in the presence and absence of 5 mM $MgCl_2$/100 μM GTPγS (single experiment observations). These data indicated a rightward shift in the dose-response curves for the muscarinic agonist carbachol and **9**. In both cases, a reduction in K_i of six-fold or greater was observed, indicating an interaction with a G-protein. Such an effect of GTPγS is typically observed in the case of muscarinic agonists and has been previously shown for the effects of carbachol on [^{3}H]QNB binding in cockroach neural tissue.[14] This provides strong evidence that compound **9** functions as a muscarinic agonist in the insect (*Musca*) tissue. Also, it is not unreasonable to infer from this observation that the other compounds in this study, previously determined to be muscarinic agonists in mammalian tissue,[20,21] function similarly in the insect tissue.

The most compelling evidence for a particular mode of action is gained when one can demonstrate a relationship between in-vitro potency for the receptor and in-vivo efficacy (mortality). Using the 16 analogs tested in this study, regression relationships between *Musca* [^{3}H]QNB binding displacement potency (K_i in μM) and efficacy (LC_{50} values in mg litre^{-1}) were

TABLE 3
Insecticidal Activity and In-vitro Receptor Binding Activity of *exo*-1-Azabicyclo[2.2.1]heptanes

			LC_{50} (*mg litre*$^{-1}$)				K_i (μM)[b]
Cmpd	*X*	*R*	*BPH*[a]	*GLH*	*TSSM*	*CA*	musca
10	O[c]	NH_2	0·3	1·4	0·2	1·6	0·095
14	CH	NH_2	4·6	29	26	85	1·47
15	N	NH_2	0·2	0·6	1·0	1·7	0·15
16	N	H	0·4	0·8	1·0	100	2·34
17	N	Br	0·1	0·2	0·5	3·3	0·028
18	N	SMe	0·7	39	56	100	4·25

[a] As Table 1.
[b] QNB Binding; data are the mean of triplicates with SEM < 15%.
[c] See Fig. 5.

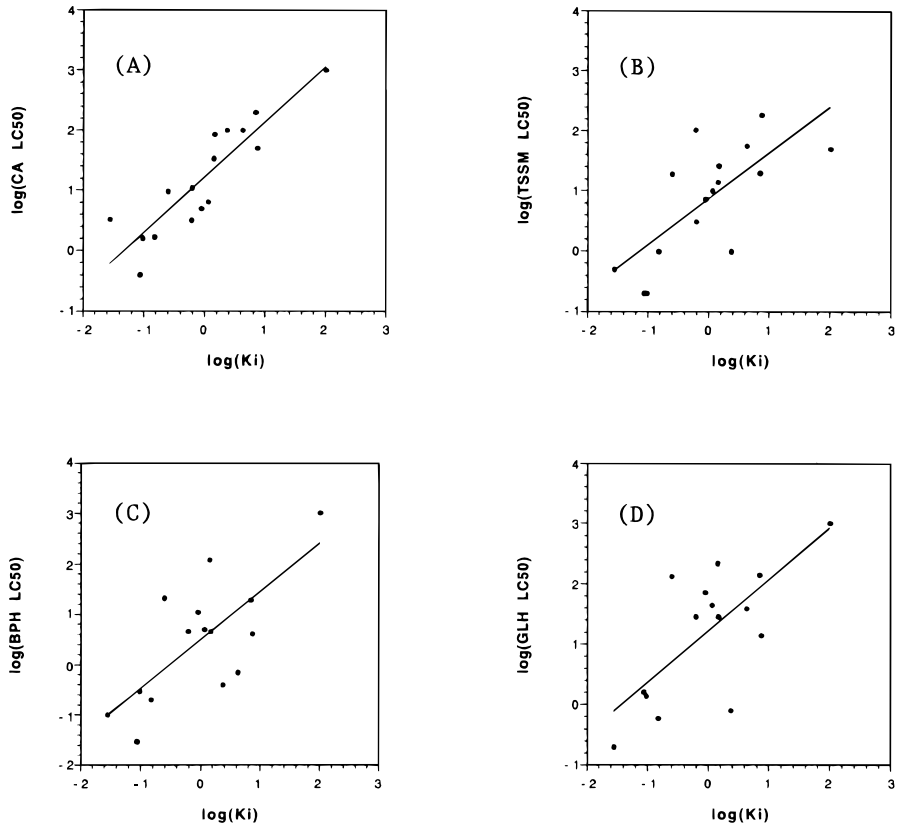

Fig. 6. Regression relationships between mAchR binding affinity and insecticidal activity. (A) CA vial assay for contact, $r^2 = 0{\cdot}79$; (B) TSSM squash cotyledon contact, $r^2 = 0{\cdot}51$; (C) BPH rice foliar contact, $r^2 = 0{\cdot}53$; (D) GLH rice foliar contact, $r^2 = 0{\cdot}51$.

determined for the three insect and one mite species (Fig. 6(a)–(d)). K_i and LC_{50} values were $\log_{10}$ transformed to stabilize the respective variances. The pyrrolidine **11** was not active against brown planthopper, green leafhopper or cotton aphid at the highest rates tested, so the LC_{50} values for these species were estimated to be 1000 mg litre^{-1}. Brown planthopper and green leafhopper LC_{50} values were not determined for compound **7** and were therefore not included in the regression analyses for these organisms. Regression analyses were performed using PROC GLM, SAS 6.09.[34]

Results of the four regression analyses are summarized in Table 4. All of the regressions were highly significant. The probability that the two sets of data were unrelated in any of the model systems was extremely low (worst case[6(d)]: $<0{\cdot}3\%$). The best regression relationship was observed between log(*Musca* [^{3}H] QNB K_i) and log(cotton aphid LC_{50}). This regression model accounted for 79% of the variation in log(cotton aphid LC_{50}). The other three regression models were somewhat less predictive, accounting for 51–53% of the variation within the respective data sets.

The fact that the regression models accounted for only 50–80% of the variation within the LC_{50} data sets is certainly reasonable considering all of the bioassays were conducted on plants. Indeed, deviations from a regression model are typical when measurements include or are dependent on a second biological system since additional variables are encountered. In this particular case, the remaining variation in LC_{50} values may

TABLE 4
Regression Analysis Results

Dependent variable	*df*	*Intercept*	*Std. error of intercept*	*Slope*	*Std. error of slope*	*Significance* ($P > F$)	r^2
log(CA[a] LC_{50})	15	1·21	0·11	0·92	0·13	0·0001	0·79
log(TSSM LC_{50})	15	0·87	0·17	0·76	0·20	0·0018	0·51
log(BPH LC_{50})	14	0·48	0·22	0·96	0·25	0·0022	0·53
log(GLH LC_{50})	14	1·22	0·20	0·85	0·23	0·0028	0·51

[a] As Table 1.

be attributable to differences between the compounds in how they interact with the plant. Factors such as affinity for the plant surface, translocation within the plant, and plant degradation can all play a role in determining the 'bioavailability' of a particular material. The actual amount of material the insect is exposed to might not be the same if plant interactions differ within a series of compounds.

The remaining variation in LC_{50} values may also reflect receptor subtype differences among the insect species. *Musca* receptors, found to be predominantly receptor subtype M_3,[15] might not have been representative for the other insect species. Variance might have been minimized if the receptors of the respective pest species had been used for this study. However, we felt that the size limitation of the mites and aphids would make these membrane preparations impractical for our purposes. We did, however, attempt to develop a crude membrane preparation for the hopper species. Unfortunately, the assay produced unsatisfactory results, largely due to a significant amount of non-specific binding.

The results of these regression analyses support the premise that the insect muscarinic receptor is a potential target site for insecticidal action. There were highly significant relationships between displacement of [^{3}H]QNB at the muscarinic receptor and whole organism toxicity to three insect and one mite species. These analyses indicated that affinity for the muscarinic receptor accounted for at least half of the variation in whole organism insecticidal activity observed for these compounds. Compounds with high affinity for the receptor were highly efficacious while compounds with moderate binding affinity caused only moderate mortality.

4 CONCLUSIONS

In the present study, several known agonists of the mammalian muscarinic receptor, were prepared and evaluated for their insecticidal potential. It was discovered that sucking insects *Nilaparvata lugens* (brown planthopper), *Nephotettix cincticeps* (green leafhopper), *Tetranychus urticae* (two-spotted spider mite) and *Aphis gossypii* (cotton aphid) were particularly sensitive to most of these compounds. Several analogs proved to be extremely active, surpassing commercial standards in some of the laboratory bioassays. Biochemical data indicated that **9** acted as a muscarinic agonist in *Musca* neural tissue. Regression analysis indicated that significant relationships exist between displacement of [^{3}H]QNB at the muscarinic receptor and whole organism toxicity to three insect and one mite species.

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REFERENCES

1. Szczepanski, C., Today's research for tomorrow's markets or: how to hit a moving target. In *Recent Advances in the Chemistry of Insect Control II*, ed. L. Crombie. Royal Society of Chemistry, Cambridge, 1990, pp. 1–16.
2. Devonshire, A. L., Biochemical and molecular biological approaches to insecticide resistance research. In *Recent Advances in the Chemistry of Insect Control II*, ed. L. Crombie. Royal Society of Chemistry, Cambridge, 1990, 17–22.
3. Otto, D., Richter, R. & Karabensch, K. H., The acetylcholine receptor as a target of insecticides. *Tagungsber-Akad.*, **274** (1989) 47–58.
4. Schroeder, M. E. & Flattum, R. F., The mode of action and neurotoxic properties of the nitromethylene heterocycle insecticides. *Pestic. Biochem. Physiol.*, **22** (1984) 148–60.
5. Bigg, D. C. H. & Purvis, S. R., Muscarinic agonists provide a new class of acaricide. *Nature* (*London*), **262** (1976) 219–22.
6. Shaker, N. & Eldefrawi, A., Muscarinic receptors in housefly brain and its interaction with chlorobenzilate. *Pestic. Biochem. Physiol.*, **15** (1981) 14–20.
7. Chiappinelli, V. A., Neurotoxins acting on acetylcholine receptors. In *Natural and Synthetic Neurotoxins*, ed. A. Harvey. Academic Press, 1993, 67–109.
8. Dudai, Y. & Ben-Barak, J., Muscarinic receptor in *Drosophila melanogaster* demonstrated by binding of [^{3}H] quinuclidinyl benzilate. *FEBS Lett.*, **81** (1977) 134–6.
9. Jones, S. W. & Sumikawa, K., Quinuclidinyl benzilate binding in house fly heads and rat brain. *J. Neurochem.*, **36** (1981) 454–9.
10. Meyer, M. R. & Edwards, J. S., Muscarinic cholinergic binding sites in an orthopteran central nervous system. *J. Neurobiol.*, **11** (1980) 215–9.
11. Breer, H., Properties of putative nicotinic and muscarinic cholinergic receptors in the central nervous system of *Locusta migratoria*. *J. Neurochem. Int.*, **3** (1981) 43–52.
12. Culotti, J. G. & Klein, W. L., Occurrence of muscarinic acetylcholine receptors in wild type and cholinergic mutants of *Caenorhabditis elegans*. *J. Neurosci.*, **3** (1983) 359–68.
13. Duggan, M. L. & Lunt, G. G., Coupling of muscarinic receptors to second messenger systems in locust ganglia. In *Neurotox 88; Molecular Basis of Drug and Pesticide Action*, ed. G. G. Lunt. Elsevier Science Publishers, 1988 245–53.
14. Orr, G. L., Orr, N. & Hollingworth, R. M., Distribution and pharmacological characterization of muscarinic-cholingeric receptors in the cockroach brain. *Arch. Insect Biochem. Physiol.*, **16** (1991) 107–22.
15. Abdallah, E. A. M., Eldefrawi, M. E. & Eldefrawi, A. T., Pharmacological characterization of muscarinic receptors in insect brains. *Arch. Insect Biochem. Physiol.*, **17** (1991) 107–18.
16. Knipper, M. & Breer, H., Subtypes of muscarinic receptors in insect nervous system. *Comp. Biochem. Physiol. C. Comp. Pharmacol. Toxicol.*, **90** (1988) 275–80.

17. Shapiro, R. A., Wakimoto, B. T., Subers, E. M. & Nathanson, N. M., Characterization and functional expression in mammalian cells of genomic and cDNA clones encoding a Drosophila muscarinic acetylcholine receptor. *Proc. Natl. Acad. Sci.*, **86** (1989) 9039–43.
18. Onai, T., Fitzgerald, M. G., Arakawa, S., Gocayne, J. D., Urquhart, D. A., Hall, L. M., Fraser, C. M., McCombie, W. R. & Venter, J. C., Cloning, sequence analysis and chromosome localization of a *Drosophilia* muscarinic acetylcholine receptor. *Fed. Eur. Biochem. Soc.*, **255** (1989) 219–25.
19. Trimmer, B. A., Current excitement from insect muscarinic receptors. *Trends in Neurosciences*, **18** (1995) 104–11.
20. Freedman, S. B., Harley, E. A., Patel, S., Newberry, N. R., Gilbert, M. J., McNight, A. T., Tang, J. K., Maguire, J. J., Mudunkotuwa, N. T., Baker, R., Street, L. J., MacLeod, A. M., Saunders, J. & Iversen, L. L., A novel series of non-quaternary oxadiazoles acting as full agonists at muscarinic receptors. *Br. J. Pharmacol.*, **101** (1990) 575–80.
21. Saunders, J. & Freedman, S. B., The design of full agonists for the cortical muscarinic receptor. In *Trends in Pharmacological Sciences Supplement: Subtypes of Muscarinic Receptors IV* (*Proceedings of 4th International Symposium of Muscarinic Receptors*), ed. R. R. Levine & N. J. M. Birdsall. Elsevier Science, Amsterdam, 1989 70–5.
22. MacLeod, A. M., Herbert, R. & Hoogsteen, K., Facile synthesis of the 4-azatricyclo[2.2.1.0]heptane system. *J. Chem. Soc. Chem. Comm.*, **00** (1990) 101–2.
23. Saunders, J., Cassidy, M., Freedman, S. B., Harley, E. A., Iversen, L. L., Kneen, C., MacLeod, A. M., Merchant, K. J., Snow, R. J. & Baker, R., Novel quinuclidine-based ligands for the muscarinic cholinergic receptor. *J. Med. Chem.*, **33** (1990) 1128–38.
24. Showell, G. A., Gibbons, T. L., Kneen, C. O., MacLeod, A. M., Merchant, K., Saunders, J., Freedman, S. B., Patel, S. & Baker, R., Tetrahydropyridyloxadiazoles: semirigid muscarinic ligands. *J. Med. Chem.*, **34** (1991) 1086–94.
25. Jenkins, S. M., Wadsworth, H. J., Bromidge, S., Orlek, B. S., Wyman, P. A., Riley, G. J. & Hawkins, J., Substituent variation in azabicyclic triazole and tetrazole based muscarinic receptor ligands. *J. Med. Chem.*, **35** (1992) 2392–406.
26. Orlek, B. S., Blaney, F. E., Brown, F., Clark, M. S. G., Hadley, M. S., Hatcher, J., Riley, G. J., Rosenberg, H. E., Wadsworth, H. & Wyman, P., Comparison of azabicyclic esters and oxadiazoles as ligands for the muscarinic receptor. *J. Med. Chem.*, **34** (1991) 2726–35.
27. Thill, D. P. & Aaron, H. S., Azabicyclic Alcohols. V. Synthesis and stereochemistry of the 1-azabicyclo-[2.2.1]octanols. *J. Org. Chem.*, **33** (1968) 4376–9.
28. Grob, C. A. & Renk, E., Untersuchungen in der chinuclidin-reihe. *Helv. Chim. Acta*, **37** (1954) 1689.
29. Abbott, W. S., Method for computing the effectiveness of an insecticide. *J. Econ. Entomol.*, **18** (1925) 265–7.
30. Raymond, M., Presentation d'un programme basic d'analyse log-probit pour micro-ordinateur. *Cah. OSTROM, Ser. Ent. med. et Parasitol.*, **23** (1985) 117–21.
31. Hamilton, M. A., Russo, R. C. & Thurston, R. V., Trimmed Spearman–Karber method for estimating median lethal concentrations in toxicity bioassays. *Environ. Sci. Technol.*, **11** (1977) 714–9. Correction: *ibid.*, **12** (1978) 417.
32. Ward, J. S., Merritt, L., Klimkowski, V. J., Lamb, M. L., Mitch, C. H., Bymaster, F. P., Shannon, H. E., Olesen, P. H., Honore, T., Sheardown, M. J. & Sauerberg, P., Novel functional M1 selective muscarinic agonists. 2. Synthesis and structure-activity relationships of 3-pyrazinyl-1,2,5,6-tetrahydro-1-methylpyridines. Construction of a molecular model for the M1 pharmacophore. *J. Med. Chem.*, **35** (1992) 4011–9.
33. MacLeod, A. M., Baker, R., Freedman, S. B., Patel, S., Merchant, K. J. Roe, M. & Saunders, J., Synthesis and muscarinic activities of 1,2,4-thiadiazoles. *J. Med. Chem.*, **33** (1990) 2052–9.
34. SAS Institute Inc., *SAS/STAT® User's Guide*, Version 6, 4th edn, vol. 2. Cary, NC: SAS Institute Inc., 1989, 846 pp.